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THE UNIVERSITY OF ALBERTA

OXIDATIVE PHOSPHORYLATION BY PEA SEEDLING MITOCHONDRIA

A DISSERTATION
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF PLANT SCIENCE
by
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EDMONTON, ALBERTA
JULY, 1960.

ABSTRACT

A mitochondrial fraction was prepared from etiolated pea seedlings that showed active oxidation and phosphorylation when succinate was used as substrate. The fraction was isolated at 10,000 x g after an initial centrifugation at 1,000 x g. Magnesium, phosphorus, substrate and enzyme were used at optimum concentrations to maintain high activity. Bovine serum albumin when added to the enzyme preparation increased oxidative activity. The high energy of ATP that was formed by phosphorylation was trapped as glucose-6-phosphate in the presence of a glucose-hexokinase trapping system. A sensitive method for determining phosphorylation was established by using radioactive phosphorus in combination with a procedure for the quantitative separation of inorganic phosphorus. Although malonate acted as a competitive inhibitor at high concentrations it was oxidized slightly at low concentrations. Dinitrophenol uncoupled phosphorylation from oxidation at 10^{-4}M. Maleic hydrazide and indoleacetic acid had practically no effect upon oxidation and phosphorylation.

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Table of Contents

Page

Introduction..1

Literature Review

I. Oxidative Phosphorylation..........................2

II. Growth Regulators...................................8

A. Maleic Hydrazide.....................................8

B. Indoleacetic Acid....................................10

Materials and Methods

a. Plant Material.......................................12

b. Fractionation Procedure..............................12

c. Manometric Methods...................................13

d. Phosphorylation......................................14

(i) Colorimetry...14

(ii) Chromatography.....................................14

(iii) Radioisotope......................................15

Results and Discussion

Enzyme Preparation......................................19

Oxidative Phosphorylation...............................35

Malonate..40

Dinitrophenol...42

Maleic Hydrazide..42

Indoleacetic Acid.......................................45

Summary...47

References..49

List of Tables

Page

I. Protocol of an experiment on oxidative phosphorylation. 18

II. The effect of sucrose concentration of the grinding and resuspending solutions on succinoxidase activity ... 21

III. Distribution of succinoxidase activity in fractions of pea seedling homogenates ... 22

IV. Nitrogen recoveries on the mitochondrial fraction and the supernatant ... 25

V. Effect of magnesium upon oxidative phosphorylation ... 38

VI. Effect of phosphorus on oxidative phosphorylation ... 38

VII. Recovery of radioisotopic phosphorus in counts per minute ... 39

List of Figures

Page

1. The effect of age of seedlings on rate of oxygen uptake 24
2. Influence of pH on rate of oxygen uptake....27
3. Effect of nitrogen concentration upon activity....28
4. Effect of substrate concentration on the rate of oxygen uptake for 1959 seed +BSA....30
5. Determination of Km for 1959 seed+BSA using the double reciprocal plot....31
6. Diagram of a chromatogram showing the separation of known compounds and mixtures of compounds to establish the activity of the trapping system....35
7. Chromatogram of the separation of glucose and G6P with ethylacetate: acetic acid: water (3:3:1) solvent on Whatman #4 paper....36
8. Chromatograms showing activie phosphorylation by the formation of G6P....37
9. Effect of malonate on oxidation and phosphorylation by pea mitochondria....41
10. Uncoupling of phosphorylation from oxidation by 2,4-DNP....43
11. The effect of maleic hydrazide on oxidation and phosphorylation by pea mitochondria....44
12. The effect of indoleacetic acid on oxidation and phosphorylation using succinate as substrate....46

INTRODUCTION

Energy, required for the regulation and function of the various plant processes, is released by the oxidation of substrate and is trapped by phosphorylation in the high energy bond of adenosine triphosphate (ATP). This energy transfer is referred to as oxidative phosphorylation. Oxidative phosphorylation was first reported for animal tissue and it has since been found to occur in plants. The oxidative phosphorylation associated with a particular step in the respiratory pathway may be used as a means of determining the effects of chemicals on plant growth. This approach has been used recently to investigate the effect of various compounds including growth regulators on plant metabolic processes.

The work reported here deals with studies on oxidative phosphorylation by pea seedling mitochondria. It describes a sensitive method for measuring phosphorylation and reports the effects of malonate, 2,4-dinitrophenol (DNP), and two growth regulators maleic hydrazide (MH) and indoleacetic acid (IAA) on oxidative phosphorylation.

LITERATURE REVIEW

I. Oxidative phosphorylation

One of the first reports of the occurence of oxidative phosphorylation in higher plants was made by Millerd (44) in 1951. It was assumed to be the oxidation of a substrate such as an organic acid and the concomitant storage of energy in the high energy bond of ATP (14). The usual method of measuring phosphorylation involved colorimetric determination of the disappearance of inorganic phosphorus (Pi) from the reaction mixture (13).

The site of oxidative phosphorylation in living organisms has been determined to be in the mitochondrial fraction (56). Different workers have reported different efficiencies for mitochondrial phosphorylation. Bonner and Millerd (13) reported P:O ratios consistently less than one. Whereas, Laties (33), using cauliflower mitochondria, obtained values greater than one with α-ketoglutarate, malate and succinate. Higher values for P:O ratios were also reported for avocado mitochondria (10). The theoretical optimal P:O ratios for Tricarboxylic acid (TCA) cycle acids are two for succinate, four for α-ketoglutarate, and three for other substrates (20, 54). Brummond and Burris (14), Conn and Young (20), and Smillie (54) have reported near theoretically optimal P:O ratios for plant mitochondria.

Some workers (13, 44) encountered difficulty in

obtaining an enzyme preparation from plant material that would give measurable phosphorylation. Laties (33) found that the nature of the extraction medium profoundly influenced the extent of phosphorylation obtained. Oxidation was affected less than phosphorylation, indicating that phosphorylation was the more labile process. Exposure of the mitochondria to hypotonic solutions uncoupled phosphorylation from oxidation. Therefore, solutions supposedly isotonic were used in grinding the tissue and resuspending the mitochondria. Various workers have reported using concentrations of sucrose that ranged from 0.25 M (10) to 0.4 M (59) and 0.5 M (54).

The incorporation of ethylenediaminetetraacetic acid (EDTA) with the grinding solution has been reported by Greengard *et al*. (27), Hackett and Haas (28), Lehninger (36) and Smillie (54) to increase oxidative activity. They assumed the increased activity to result from the removal of inhibitory ions by chelation.

Mitochondrial fractions prepared by differential centrifugation of plant homogenates have been a common source of enzyme systems. These preparations have been able to oxidize TCA cycle intermediates such as pyruvate, citrate, α-ketoglutarate, fumarate, succinate, and malate. Of all the above mentioned substrates, succinate was oxidized most rapidly (10, 20, 27, 59).

The succinoxidase system involves the oxidation of succinate to fumarate with the transfer of electrons through a flavoprotein, cytochrome, and cytochrome oxidase to oxygen (18). Energy released by the passage of electrons to oxygen is utilized to form the high energy bond of ATP. The phosphorylation with succinate bypasses the diphosphopyridine nucleotide (DPN) phosphorylation step, thus only two moles of Pi are esterified per mole of substrate oxidized (37) as represented by the following diagram.

$$\begin{tikzcd}
 & & \text{Succinate} \arrow[d] & & & \\
\text{Substrate} \arrow[r] & \text{DPN} \arrow[r] \arrow[d] & \text{FAD} \arrow[r] & \text{cyt b} \arrow[r] \arrow[d] & \text{cyt c} \arrow[r] & {} \\
 & \text{ATP} & & \text{ATP} & &
\end{tikzcd}$$

$$\begin{tikzcd}
\text{cyt a} \arrow[r] \arrow[d] & \text{cyt } a_3 \arrow[r] & H_2O \\
\text{ATP} & &
\end{tikzcd}$$

In oxidative phosphorylation studies, it is desirable to work with a single step process (35). With α-ketoglutarate as the substrate, one step oxidation is achieved by the use of malonate to inhibit succinoxidase activity. When succinate is used as substrate, its concentration may be increased so that the oxidation of fumarate to other intermediates becomes negligible (27) and succinate oxidation then proceeds as a single step process (20).

Smillie (55) carried out extensive studies on succinic dehydrogenase. He investigated oxidative phosphorylation activity in particulate fractions from etiolated pea

leaves and found that the fractions containing the highest activity were sedimented at 2700 x g and 6700 x g. Similar work with green pea leaves indicated that the fraction containing succinic dehydrogenase activity was sedimented mostly at 2700 x g while the fractions sedimented at 2700 x g and 6700 x g exhibited the majority of the oxidative and phosphorylation activity (56). Pierpoint (50) also reported that oxidative phosphorylation was associated with the mitochondrial fraction.

Pierpoint (50) working with tobacco mitochondria and Hovenkamp (20) with Azotobacter vinelandii mitochondria observed a decline in activity during short term storage of the material. The P:O ratio of particulate fractions held overnight at 0° - 4° C, decreased by 10% to 50% and particulates held at 30° C for ten minutes lost 50% of their activity (20).

Mitochondria required the addition of certain cofactors to oxidize substrates rapidly. The most common cofactors that have been added are magnesium and ATP. However, depending on the nature of the extraction medium and the substrate oxidized, other cofactors were required. Biale (10) observed that avocado mitochondrial preparations required magnesium ions for rapid oxidation of succinate. This was in agreement with results obtained when other plant material was used (33, 58, 59, 62). Substrates affected by the addition of magnesium and ATP were citrate (22), α-ketogluturate (10, 33, 59), malate (10, 33, 59), pyruvate (44), and succinate

(10, 33, 59). Although ATP increased oxidative activity, adenosine monophosphate (AMP) or adenosine diphosphate (ADP) were required as acceptor molecules for the esterification of inorganic phosphate (44, 59).

Cytochrome c added to the reaction mixture was also found to increase oxidative activity in some cases. However, Biale(10) observed that cytochrome c had no effect upon avocado mitochondria. He considered the lack of effect from cytochrome c to be an indication that the mitochondrial complex was isolated in a relatively intact form. Some preparations showed a requirement for cytochrome c when pyruvate or succinate were used as substrates while α-ketoglutarate oxidation did not require the addition of cytochrome c (59).

The activity of enzyme systems from mitochondrial preparations was observed to increase upon the addition of an inert protein, bovine serum albumin (BSA). Wojtczak and Wojtczak (65) reported that bovine and human serum albumin increased P:O ratios while egg albumin was ineffective. An increase in oxidative phosphorylation was also reported by Hovenkamp (29) working with extracts of Azotobacter. Rat liver mitochondria lost the ability to oxidize succinate when they were irradiated with ultraviolet light (9), but the incorporation of BSA prevented the detrimental effect of the ultraviolet bands. In the above cases, BSA tended to increase oxidative activity of succinoxidase and increased P:O ratios by increasing the efficiency of phosphorylation. However, Greengard _et al._

(27) reported that BSA did not affect succinate oxidation.

The rate of oxidation of TCA cycle intermediates by mitochondria is affected by the acidity of the reaction mixture. Millerd et al. (44), using mung bean conducted their experiments at a pH of about 7.1 and Laties (33) working with lupine, did his at pH 7.3. Succinoxidase activity of mitochondria has been observed (59) to be equal at pH 7.1 and pH 6.9, however, the activity decreases at pH 7.3. Pierpoint (50) working with tobacco leaf mitochondria found that succinoxidase activity was optimum in the range pH 6.8 to pH 7.2. Utter et al. (62) working with a mitochondrial preparation from yeast, observed that oxidative phosphorylation rates are optimum between pH 6.6 and pH 6.9. All of these studies suggest that the succinoxidase system is most active at neutral or slightly acidic pH.

Fluoride has been used quite commonly in oxidative phosphorylation studies to inhibit ATP hyrolysis (28). This makes it possible to measure the Pi esterified. However, Conn and Young (20) reported that in the presence of a glucose - hexokinase trapping system, the effects of fluoride are minimized. The studies by Switzer and Smith (59) and Utter et al. (62) also bear this out since fluoride had no effect when they used a glucose-hexokinase trapping system.

Another inhibitor used in oxidative phosphorylation studies of TCA cycle acids is malonate. It is a specific inhibitor of succinic dehydrogenase and is used in kinetic

studies of this enzyme (11, 23). When α-ketoglutarate is the substrate, malonate is used to inhibit succinate oxidation giving a single step process (20, 50).

The use of 2,4-dinitrophenol (DNP) for uncoupling of phosphorylation from oxidation has been demonstrated (40). The uncoupling action of DNP has also been demonstrated with plant mitochondria by Bonner and Millerd (13), and Laties (34). Usually an increase in the rate of oxidation is accompanied with uncoupling. However, this is not always the case (20, 30). Romani and Biale (53) working with avocado mitochondria found no increase in oxidation at 10^{-5} M DNP, while phosphorylation was uncoupled. The concentration required for uncoupling of phosphorylation has been in the order of 10^{-5} M to 10^{-4} M, (24, 28, 32, 33).

II Growth Regulators

A. Maleic Hydrazide.

The first reports of plant growth regulator activity of MH were relatively recent. It has been used to a limited extent as a herbicide. MH is effective in controlling a few perennial grasses, and when applied as a preharvest foliar spray to potatoes and onions, sprout formation is inhibited during storage (66).

Little is known about the mode of action of MH. One of the theories suggested was that MH acts as a hormone-like growth regulator (21). Leopold and Klein (38) proposed that MH is an antiauxin. They observed that MH, at low concentration

showed no effect in a straight growth test, while at high concentration growth was inhibited. Indole acetic acid was observed to overcome MH inhibition at some concentrations.

The effects of MH on enzyme systems, especially the dehydrogenases, were studied and MH was suggested to affect respiration by affecting the dehydrogenase enzymes (31). Dehydrogenase activity has been inhibited in soluble and mitochondrial systems at 4.5×10^{-4} M to 3×10^{-3} M and this effect has been proposed to be through the -SH groups of the enzymes (66).

Although the plant metabolic pathways affected by MH have not been ascertained it has been suggested that carbohydrate metabolism is altered. The observation of exudates of sucrose on treated plants was considered as evidence for this. The possibility that MH affected plant growth through the influence on phosphorus metabolism was also proposed (66).

Muir and Hansch (46) studied the problem of thiol reaction with MH and proposed a chemical explanation of a mechanism whereby MH could inhibit growth. They suggested that MH was able to react with the free thiol groups on the enzyme and thus inhibit growth. Weller _et al_. (64) studied the problem of MH reaction with thiol groups and found no evidence that MH could combine or react with them. The evidence for the structure of MH being in the form of a phenol-type hydroxyl (21, 43) would make the reaction of MH and thiols unlikely (64).

B. Indoleacetic Acid

Indoleacetic acid, a universal plant growth regulator, has been isolated from both higher and lower plant forms (12). IAA is produced in the meristimatic region of the growing point and the downward movement regulates stem elongation, root growth, and growth of auxiliary buds (60).

IAA has been observed to affect growth elongation and increase plant respiration. Burström (17) reported that conditions of the cell wall are very important in growth and that IAA may alter the structural properties to permit increased growth. Workers generally agree that the affect of IAA upon the elongation of isolated tissue is primarily due to the increase in water uptake. This is presumed to result from the increased permeability of the cell wall (12). Some theories of changes in the composition of the cell wall, expecially pectic substances, have been presented. Using in vitro studies Bryan and Newcomb (15) reported an increase in the pectin methylesterase activity.

Secondary effects of IAA upon overall growth have also been suggested to occur by the action of IAA in intracellular metabolism. Marrè suggested that IAA facilitates the reduction of glutathione by ascor bic acid (41). Chinoy (19) also reported interaction between IAA and ascorcbic acic metabolism.

Attempts to determine the mode of action of IAA

have been associated with studies of synergistic and antagonistic effects of other growth regulators and also the effects of ions, such as Mn, K, Ca, and Co, upon growth (7).

Attempts to determine the mode of action of IAA are hampered by the difficulty of observing or examining a cause that is due to a primary effect. Since growth is a complexity of many processes, the difficulty in observing the primary effect is amplified (7).

MATERIALS AND METHODS

(a) Plant Material

Peas (*Pisum sativum*)variety Little Marvel (1958 or 1959 seed) were treated with 1% orthocide, soaked in distilled water and planted in moist autoclaved vermiculate in glass trays. The material was grown in the dark in a growth cabinet at 18° C.

(b) Fractionation Procedure

Following is a description of the solutions used in fractionation and the procedure employed. All steps were carried out at 0° - 4° C.

Solution I - 0.44 M. sucrose, 0.01 M. phosphate buffer at pH 6.90.

Solution II - solution I with 3 mg. per ml. BSA.

Three to four days old etiolated pea seedlings were ground in solution I, using a Serval Omnimixer at half maximum voltage.

Seedlings (50 gm.) ground in 100 ml. solution I, filtered through cheesecloth.
↓
Homogenate
1,000 x g.
10 min.
↓
Crude Homogenate + sediment (starch, cell debris, chloroplasts)
10,000 x g. discard
20 min.
↓
Supernatant + brown sediment (mitochondria)
discard
homogenized in 10 ml. of solution II in a glass tissue homogenizer.

The enzyme preparation was held at 0° C. in a mixture of ice and water and used as soon as possible.

(c) Manometric Methods

Oxygen uptake was measured by the conventional Warburg Method (61) at 30° C. Vessels of approximately 17 ml. volume were used and the total volume of the reaction mixture was 2.2 ml., which included magnesium, ATP, phosphate buffer, enzyme, and 20% KOH in the center well. The volume was increased when accompanying phosphorylation was measured. Additions were magnesium, AMP or ADP, phosphate buffer, a glucose-hexokinase trapping system, enzyme, and KOH, in the center well. All components were chilled and the enzyme was added to the chilled vessels.

When 2, 4-DNP or malanate were tested, the enzyme

and chemical were added to the sidearm to allow equilibration for 10 minutes. After equilibration, the contents of the sidearm were mixed and rinsed once with the contents of the main compartment. Readings were begun 2 minutes after mixing. When the sidearm was not used, a 10-minute equilibration period was allowed. The rate of oxygen uptake was linear for the first hour. However, all calculations were based on the first 21 minutes and were expressed as µl of oxygen mg.N/hr.

Nitrogen was determined by a micro-Kjeldahl method (4). The nitrogen added in the form of BSA was deducted from the nitrogen content of the enzyme preparation.

(d) Phosphorylation

Three methods for phosphorus determination were attempted.

(i) Colorimetry

Waygood's adaptation (63) of the Lowry and Lopez method and Martin and Doty's (42) method were used. The major shortcomings of both methods was their lack of sensitivity. Consequently, they were abandoned.

(ii) Chromatography

Methods of Bandurski and Axelrod (6), and Mortimer (45) were compared for the separation of glucose and G6P by ascending paper chromatography. The chromatography was carried out on either strips or sheets of Whatman #4 filter paper at room temperature (approx. 23°C.). The solvents were methyl

alcohol: ammonium hydroxide: water (6:1:3), methyl alcohol: formic acid: water (80:15:5) and ethyl acetate: acetic acid: water (3:3:1). The second solvent had to be filtered to remove a fine white precipitate that usually formed. Glass-distilled ethylacetate and water were used for the third solvent as described by Mortimer.

The chromatograms were run without equilibration and good separation was obtained by irrigating the chromatograms twice with complete drying between irrigations.

The dried chromatograms were sprayed first with Partridge reagent (49) and were placed in an oven at 105° C. for five minutes to develop. Glucose and G6P showed as brown spots. The second spray for the detection of Pi, ATP and ADP was a modification of Burrow's spray reagent (16). The perchloric acid was replaced with an equal concentration of HCl to prevent the destruction of the chromatograph. The chromatograms developed at 50-60° C. for 15-20 minutes showed blue spots on a lighter blue background. Exposure to concentrated ammonia fumes reduced the blue background to a tan colour with the spots remaining blue.

(iii) Radioisotope

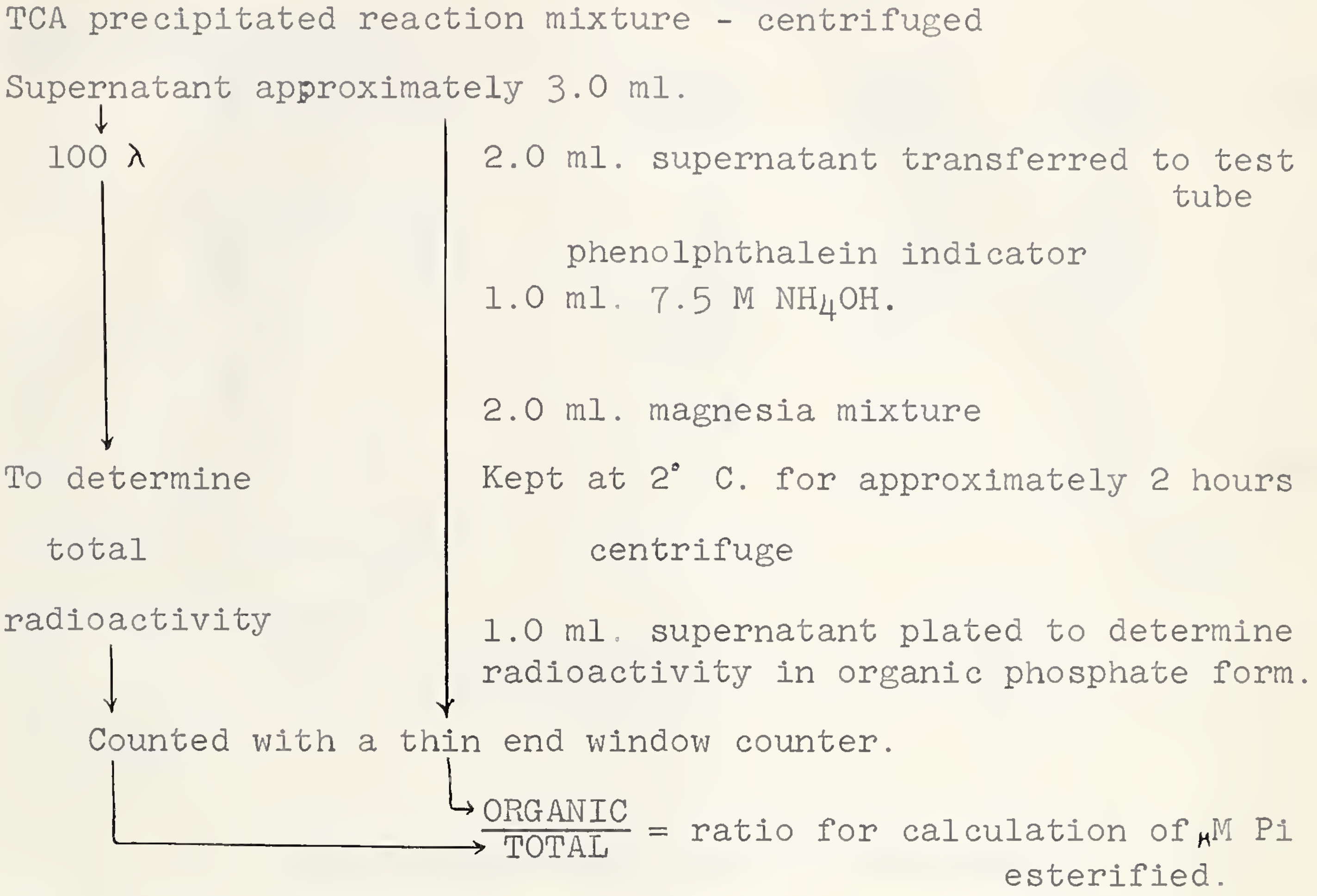
A radioisotopic method (3) was used for the quantitative estimation of phosphorylation. These studies were carried out in 25 ml. Erlenmyers on a Dubnoff metabolic shaker at 30° C. The reaction mixture was the same as for manometric studies except that the volume was reduced to 3 ml.

since KOH was not required.

Radioactive phosphorus was added to the reaction mixture after a ten minute equilibration period. Approximately 50,000 to 80,000 cpm were added in a volume of 0.1 ml. and the time of addition was taken as initial or 'zero' time. After 21 minutes, 0.3 ml. of 20% trichloroacetic acid (TCA) was added to stop the reaction. The contents of the Erlenmyers were transferred to test tubes and the TCA precipitate was brought down by centrifugation. The supernatant was analyzed for total radioactivity by plating a 100 λ sample in a stainless steel sample cup. A few drops of dilute detergent were added to spread the sample in a thin uniform film over the area in the cup. The samples were dried under an infrared lamp and were counted.

After TCA precipitation, a 2.0 ml. aliquot of the supernatant of each sample was transferred to another test tube and treated with magnesia mixture (61). This brought the volume to 5 ml. of which a 1.0 ml. sample was counted for organic phosphorus.

The following procedure was used:



TCA precipitated reaction mixture - centrifuged

Supernatant approximately 3.0 ml.

100 λ

To determine total radioactivity

2.0 ml. supernatant transferred to test tube

phenolphthalein indicator

1.0 ml. 7.5 M NH_4OH.

2.0 ml. magnesia mixture

Kept at 2° C. for approximately 2 hours

centrifuge

1.0 ml. supernatant plated to determine radioactivity in organic phosphate form.

Counted with a thin end window counter.

$\frac{\text{ORGANIC}}{\text{TOTAL}}$ = ratio for calculation of μM Pi esterified.

Sufficient NH_4OH was required to make the reaction basic to phenolphthalien. The magnesia quantitatively precipitated Pi as $MgNH_4PO_4 \cdot 6H_2O$ leaving the organic phosphates in solution. Addition of more magnesia mixture to the organic portion did not precipitate any additional Pi. The radioactivity in the organic form divided by the total radioactivity gave a ratio, which multiplied by the total Pi in the reaction mixture, gave the amount of Pi esterified in μM.

The data for a type experiment on oxidative phosphorylation are presented in detail in table I.

Table I. Protocol of an experiment on oxidative phosphorylation.

	Endogenous		Reaction	
Total radioactivity				
cpm / 100 λ	2283	2963	3888	2705
cpm / 2.0 ml.	45660	59260	77760	54100
Organic radioactivity				
cpm / 1.0 ml.	43	48	2318	1537
cpm / 5 ml.	215	240	11590	7685
Ratio org./total	0.005	0.004	0.149	0.142
Ratio $_{x}$66 MPi*	0.33	0.26	9.83	9.37
μMPi / mg.N./ hr.**	0.65	0.51	19.37	18.46
Average of duplicates	0.58		18.92	
Net esterification	18.34			
Net oxygen uptake (μgram atom O_2)	14.28			
P:O ratio	1.28			

*The concentration of Pi in the reaction mixture which multiplied by the ratio gave the μMPi esterified per vessel for 21 minutes.

$$**\frac{\text{Pi esterified per vessel per 21 minutes}}{\text{Mg. Nitrogen per vessel (1.45)}} \times \frac{60}{21} .$$

The radioisotope method provided a sensitive and relatively simple means of measuring oxidative phosphorylation.

RESULTS AND DISCUSSION

Enzyme Preparation

The oxidative activity and to a greater extent the phosphorylative activity were found to be labile. The mitochondria lost activity very rapidly upon standing. Storing the enzyme preparations at 0° C resulted in the loss of 34% of the succinoxidase activity during the first hour and 17% during the following three hours. The loss of activity of the enzyme preparation that was frozen 24 hours at -20° C was only 19%. Storage of the enzyme preparation for longer periods of time resulted in a continued loss of activity. Studies with material prepared and frozen for two hours at -20° C indicated that the initial rate of activity was the same but the total uptake at one hour was somewhat lowered.

To keep the loss of activity at a minimum, all additions to the Warburg vessels were complete before the enzyme was in the final stages of isolation. Immediately after the enzyme was prepared, the required amounts were added to chilled vessels and the experiment commenced.

The methods already described were adopted because they made it possible to prepare the enzyme at a low temperature and in a minimum of time. When the tissue was homogenized for less than one minute the activity remained high whereas grinding for one to two minutes decreased enzyme activity. Biale (10) suggested that the decreased activity following continued homo-

genization at high speeds may be due to damage to the mitochondria or the oxidation of the thiol groups of the enzyme.

Biale (10) and Laties (33) stressed the importance of using an isotonic grinding medium. However, since their supposedly isotonic solutions ranged in concentration of sucrose, it was desirable to determine the tonicity required to obtain high activity with the succinoxidase system.

Table II shows the effect of sucrose concentration on succinoxidase activity. The activity was the highest when 0.44 M sucrose was used in the grinding and resuspending solutions. It was lowest when 0.2 M sucrose was used.

The distribution of succinoxidase activity in the fractions of pea seedling homogenates is shown in table III. The data show that the mitochondrial fraction contained all the activity while none was present in the supernatant fraction. Experiment B1 indicates that at 10,000 x g all the succinoxidase activity was isolated. Experiment B2 was carried out to determine the activity of the fraction between 10,000 x g and 20,000 x g. The results indicate only 25% of the activity present in that fraction.

The centrifugal force of 20,000 x g. used in the preliminary studies corresponds to the methods of preparation of avocado and cauliflower mitochondria by Biale (10) and Laties (34). However a large proportion of green material, assumed to be chloroplast fragments, was sedimented with the mitochondria at

Table II. The effect of sucrose concentration of the grinding and resuspending solutions on succinoxidase activity.

Concentration of Sucrose		Rate of Uptake $QO_2(N)$	Total uptake in 90 min. ulO_2
Grinding Solution	Resuspending Solution		
0.2 M	0.2 M	44	61
0.2 M	0.44 M	74	69
0.44 M	0.2 M	123	196
0.44 M	0.44 M	213	205

Table III. Distribution of succinoxidase activity in fraction of pea seedling homogenates.

Experiment No.	Fraction	Force xg	Activity Q_{O_2}(N)
A	Crude homogenate	1,000	12
	Mitochondrial	20,000	71
	Supernatant	20,000	nil
B I	Crude Homogenate	1,000	39
	Mitochondrial	10,000	262
	Supernatant	10,000	nil
2	Crude homogenate	1,000	not tested
	Mitochondrial I	10,000	183
	Mitochondrial II	20,000	47
	Supernatant	20,000	nil

Reaction Mixtures.

A ATP - 0.5μM; $MgSO_4$ - 1μM; Succinate - 20μM; phosphate - 29μM; Sucrose - 160μM. Total Volume - 2.0 ml.

B I AMP - 1μM; $MgSO_4$ - 1μM; Succinate - 20μM; phosphate - 12μM Hexokinase - 70 KM units; glucose - 8μM; BSA - 0.6mg/ml; Total volume - 3.0 ml.

2 AMP - 1μM; $MgSO_4$ - 1μM; Succinate - 20μM; phosphate - 22μM Hexokinase - 70 KM units; glucose -μ8 M; BSA - 0.6mg/ml; sucrose - 88μM. Total Volume - 3.0 ml.

20,000 xg. Smillie (55, 56) used relatively low forces of gravity (6700 xg) to isolate the mitochondria from etiolated and green pea leaves. In revising the method of mitochondrial isolation it was observed that at 10,000 xg the mitochondrial fraction was relatively free of green material while the fraction between 10,000 xg and 20,000 xg contained a very large proportion of it.

The activity of the succinoxidase system of pea seedlings of various ages was studied. Fig. 1 shows the results that were obtained. The activity increased rapidly to a maximum at three to four days and decreased by the fifth day. The curve for the 1958 seed was obtained in the preliminary studies but the sharp peak on the fourth day was objectionable as slight variations in the age of the tissue caused a large decrease in activity. This condition was changed to a considerable entent by the use of BSA during mitochondrial isolation as is shown by the 1958+BSA curve. The maximum activity was maintained for a longer period of time and the variations were not as great. However, the maximum was now at three days. The 1958 seed + BSA was used for all studies except where otherwise stated.

The results of the 1959 seed + BSA were similar to the 1958 seed + BSA except the maximum was again shifted to four days. The results obtained were in general agreement with those obtained by other workers, (1, 26).

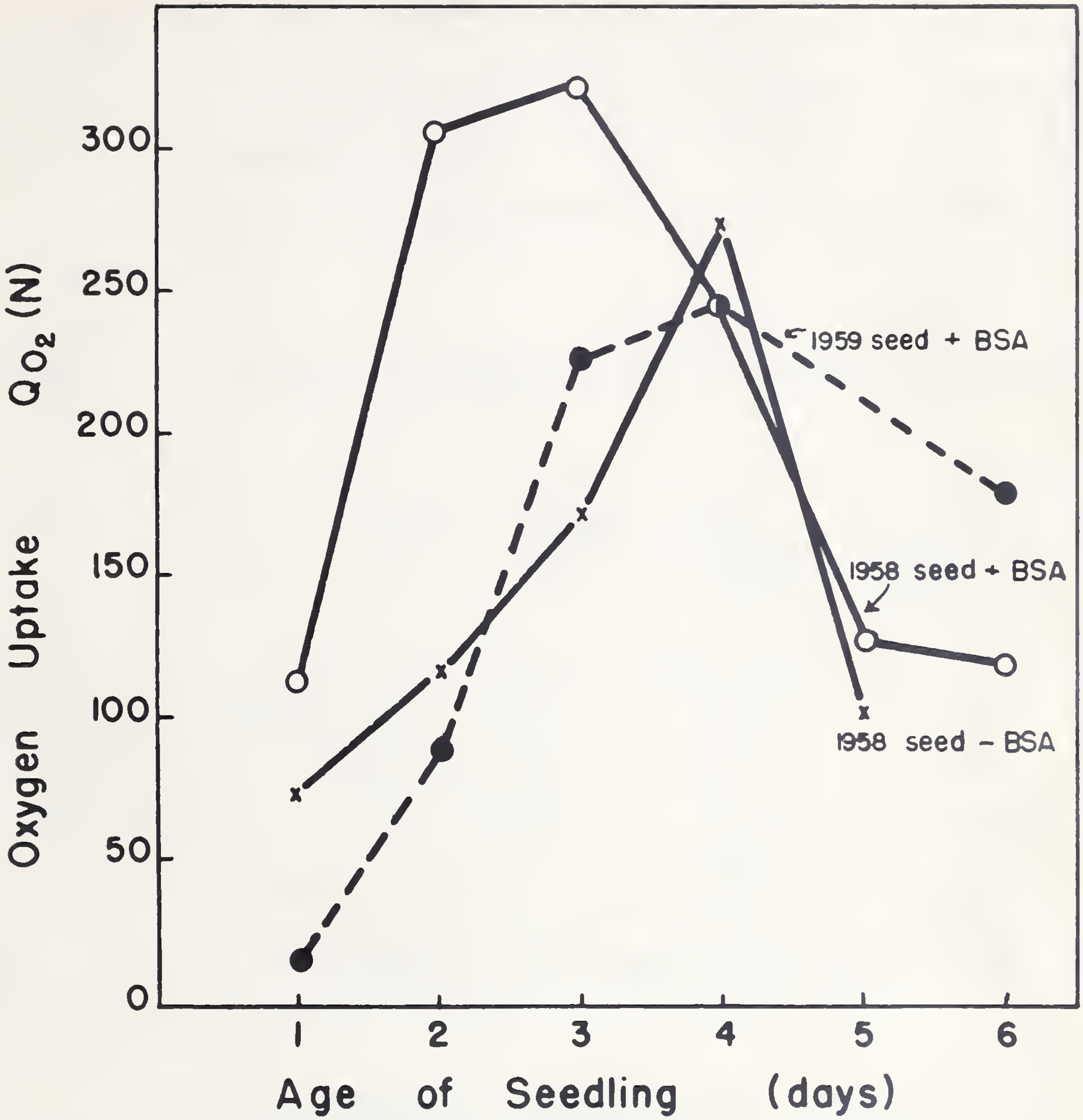


Fig. 1. The effect of age of seedlings on the rate of oxygen uptake.

The results of the nitrogen recoveries are shown in table IV. The majority of the nitrogen remained in the supernatant and approximately 10% of the nitrogen was isolated in the mitochondrial fraction. Similar work by Pierpoint (50) indicated that tobacco mitochondria contained 15% of the total nitrogen.

Table IV. Nitrogen recoveries on the mitochondrial fraction and the supernatant.

Fraction	Volume ml.	Nitrogen mg/ml.	Total nitrogen in mg.	Percent of Crude Homogenate
Crude homogenate*	50.0	3.10	155.0	
Supernatant	48.2	2.82	135.9	88%
Mitochondria	7.5	2.05	15.5	10%
Nitrogen recovered			151.4	98%

*50 ml. of crude homogenate centrifuged. Mitochondria suspended in 5 ml. solution II.

The following observations are apparent from a comparison of tables III and IV. The activity of the crude homogenate is extremely low even though it contains all the nitrogen. The supernatant, which contains a very high proportion of the nitrogen is inactive. The low nitrogen concentration and the high activity of the mitochondrial fractions is an indication that the succinoxidase activity is localized in the mitochondria.

The addition of BSA to the enzyme preparation had a stabilizing effect upon the activity of the pea succinoxidase. Factors taken as evidence for the increased stability were the improved reproducibility of activity from one preparation to another, the extended linearity of reaction rate, and the increase in total oxygen uptake. The activity of the succinoxidase, when BSA was omitted, was quite unstable and the initial rate was linear for about 5 minutes after which time there was a drop in the rate of activity.

The explanation for BSA protection of the mitochondrial succinoxidase is not definitely known. Hovenkamp (29) reported that BSA stabilized the oxidative phosphorylation activity of extracts of Azotobacter vinelandii, however he was unable to explain this protective action. Similarly Beyer (9) and Wojtczak (65), have observed the protective action of serum albumin. Polis and Shmukler (51) have suggested that during irradiation by ultra-violet light an inhibitor may be released which is bound by the albumin. If during enzyme preparation an inhibitor was released, it might be bound by the BSA and increase oxidative and phosphorylative activity. Another explanation of BSA action has been suggested by Stern and Timonen (57). They reported that serum albumin seemed to oppose the destructive effect of dilution upon the mitochondrial activity.

For pH studies, a dilute phosphate buffer was used during mitochondrial isolation. It represented only 10% of the

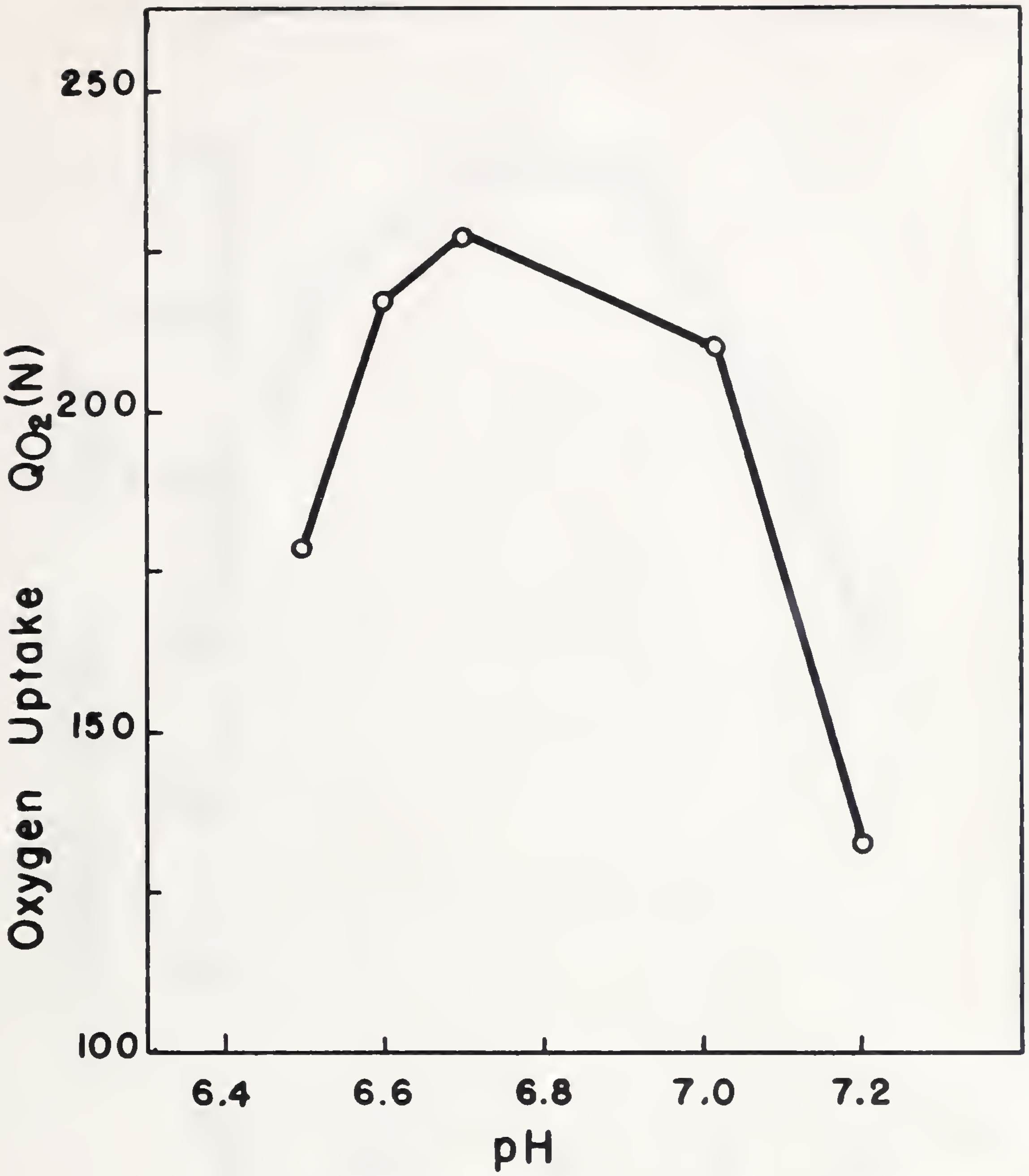


Fig. 2. Influence of pH on rate of oxygen uptake.

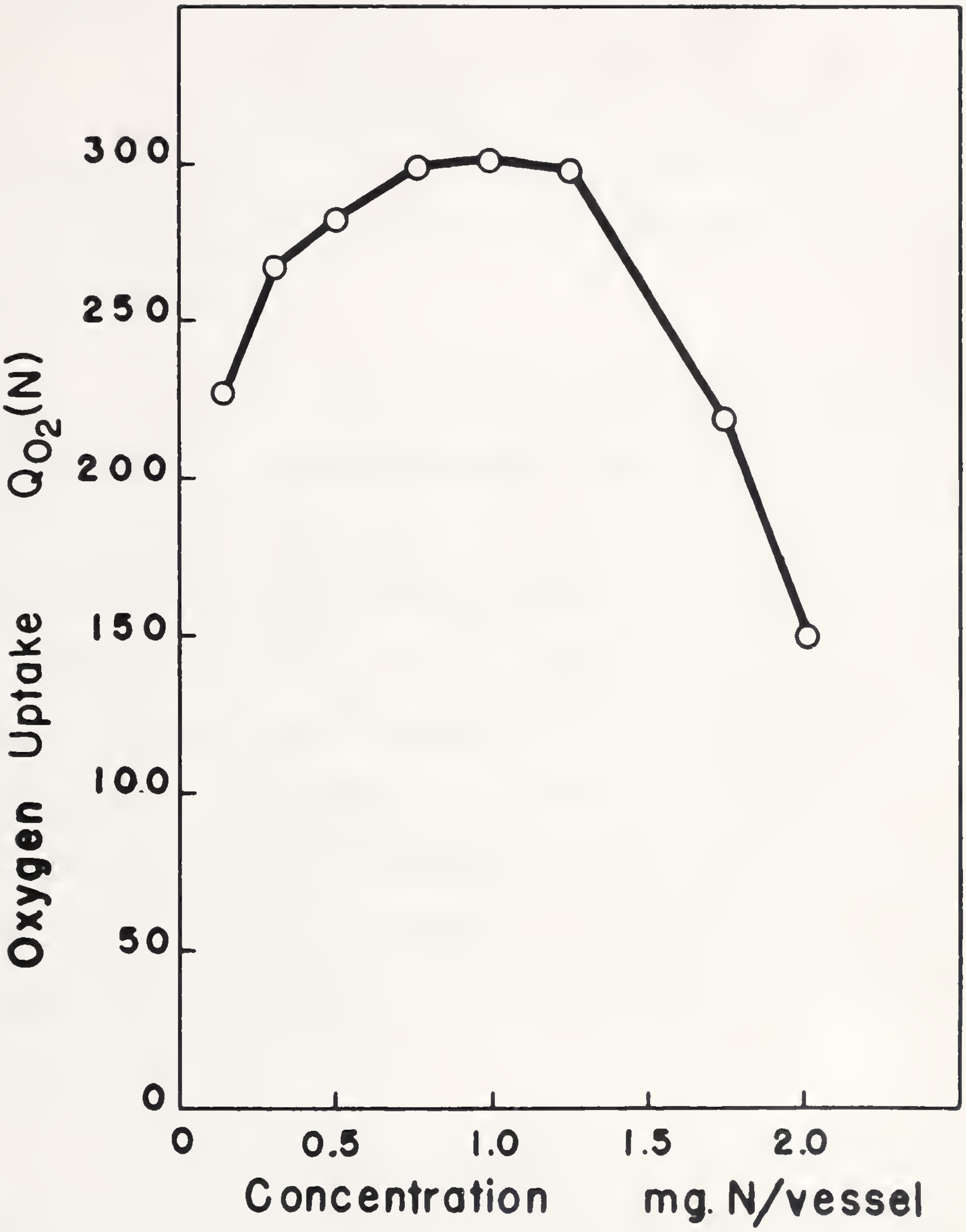


Fig. 3. Effect of nitrogen concentration upon activity. Reaction contained 20 µM substrate for 1958 seed+BSA and 50 µM for 1959 seed+BSA.

buffer. The remaining 90% of the buffer was added at the required pH levels, to maintain the pH in the reaction mixture.

The succinoxidase system was most active between pH 6.6 and pH 7.0. The activity dropped off very rapidly towards the basic side and at pH 7.2 approximately half of the activity was lost. (Fig. 2).

Preliminary studies indicated that the activity of various preparations varied with their nitrogen concentration. The results of experiments set up to test the effect of enzyme concentration on the succinoxidase activity are shown in Fig. 3. The highest activity was in a range from 0.75 to 1.25 mg. N per vessel. Using fresh material for each experiment introduced variations in the nitrogen concentration which were negligible unless the nitrogen concentration was below 0.3 mg. N per vessel or above 1.5 mg. N per vessel. The increase in nitrogen concentration above 1.5 mg. N resulted in a lowered and very unsteady reaction rate.

The optimum substrate concentration for preparations from 1958 seed + BSA was 0.01 M and was 0.05 M for 1959 seed + BSA. Increase in the concentration of substrate considerably above optimum resulted in inhibition of the rate of activity, (Fig. 4). The most suitable concentration to use in the studies was in or approaching the plateau region of the curve, as slight variations in the amount of substrate added had little effect on the rate of activity. To insure that substrate was not rate limiting, the concentration was increased

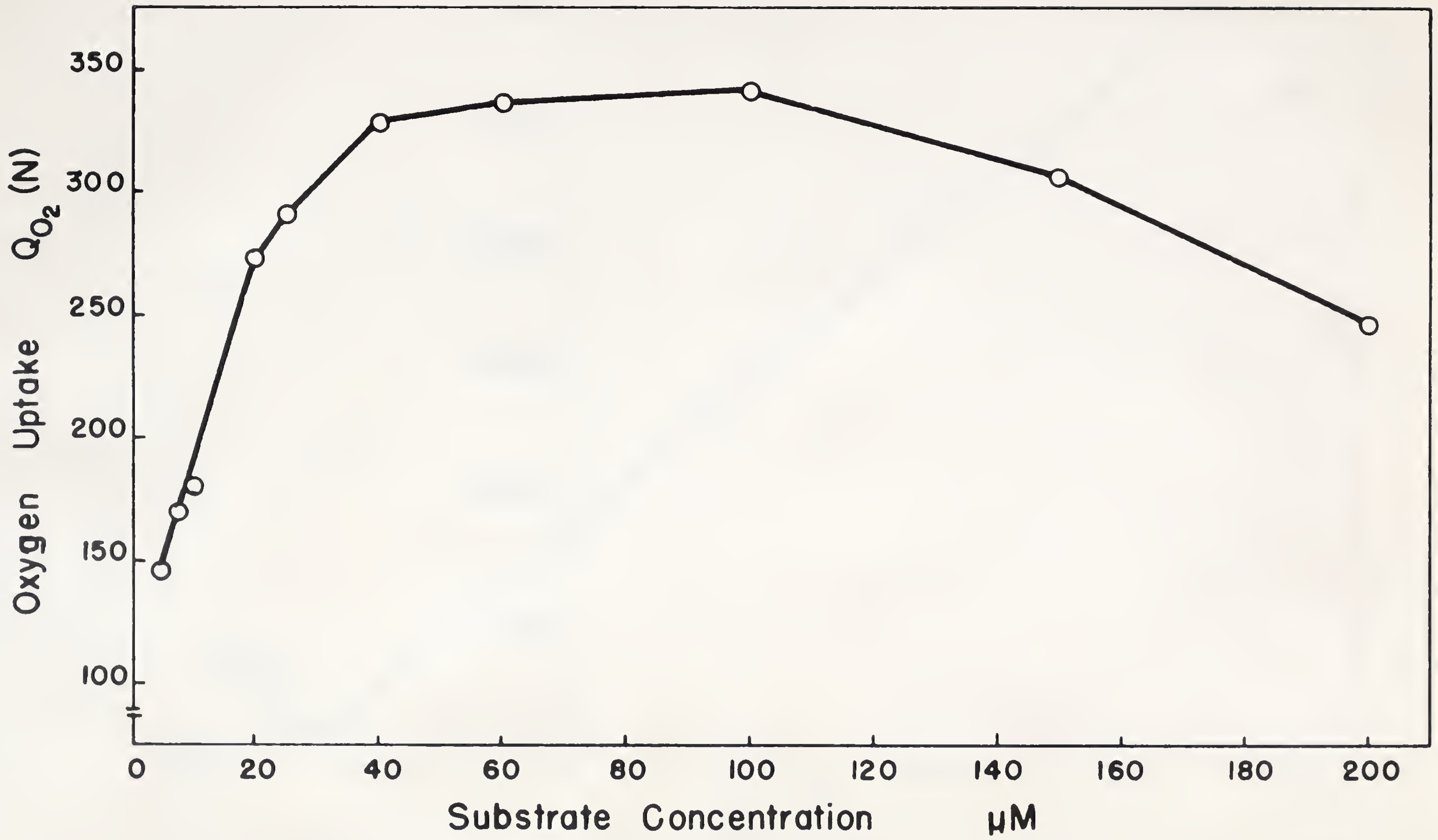


Fig. 4. Effect of substrate concentration on the rate of oxygen uptake for 1959 seed+BSA.

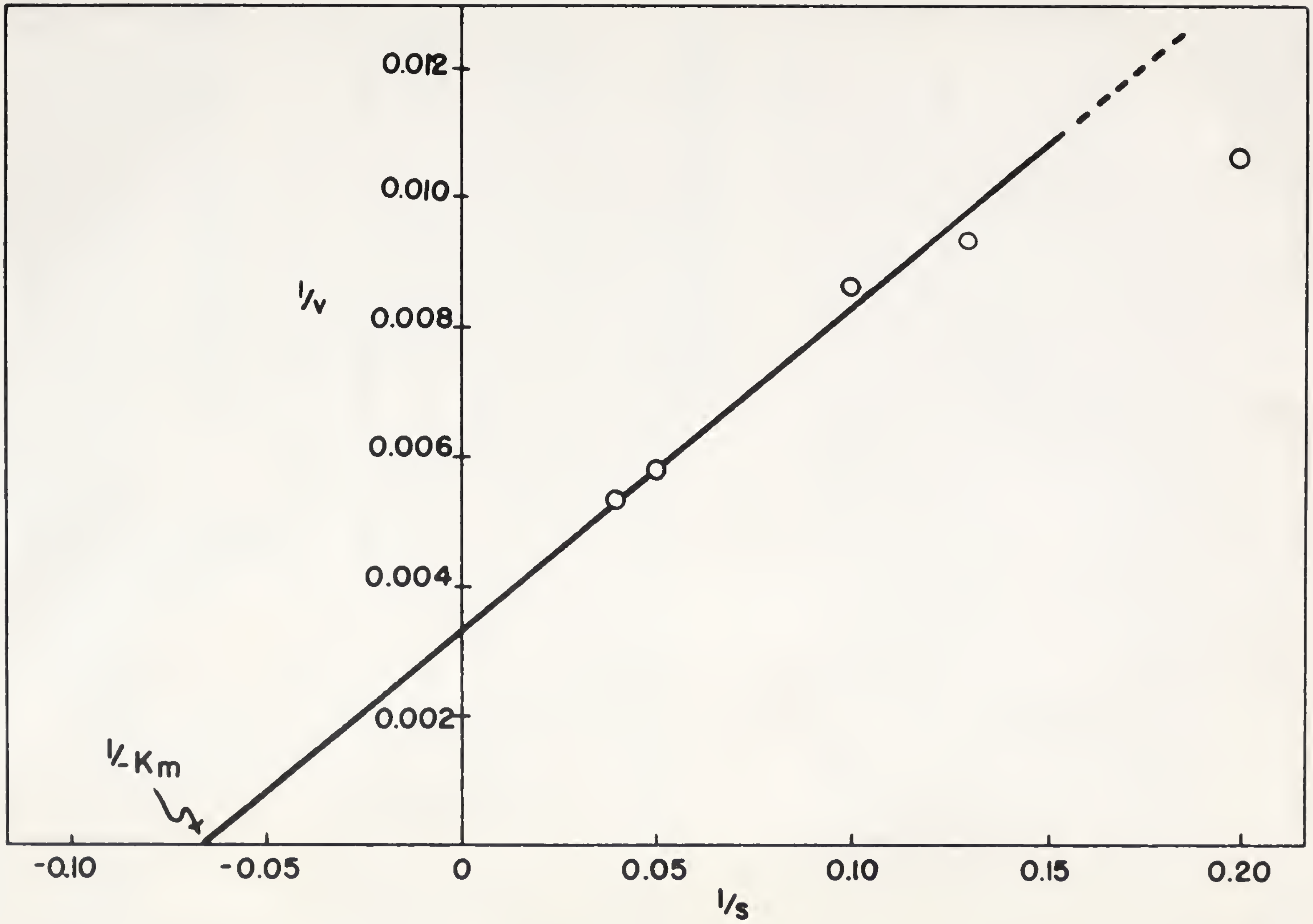


Fig. 5. Determination of Km for 1959 seed+BSA using the double reciprocal plot. Substrate concentration s in µM, and v is rate of oxygen uptake.

Km = 0.014 M.

somewhat above optimum. This increase did not alter the optimum activity.

The Michaelis constant (Km) was determined on the preparations from both 1958 and 1959 seed + BSA. The Km value for the 1958 seed + BSA was 0.005 M and 0.014 M for 1959 seed + BSA. The Lineweaver-Burk(39) double reciprocal plot was used to determine Km (Fig. 5.). This involved the rearrangement of the Michaelis-Menten equation and plotting the reciprocal of the rate ($\frac{1}{v}$) against the reciprocal of the substrate concentration ($\frac{1}{s}$).

Oxidative Phosphorylation

Phosphorylation is the process whereby the energy released by oxidation is trapped in the form of high energy phosphate bonds of ATP. For this step a phosphate acceptor such as AMP or ADP is required. Several experiments were designed to compare the suitability of AMP and ADP as phosphate acceptors. The following data show that ADP increased phosphorlation with a very slight effect upon oxidation.

	AMP	ADP
phosphate esterified (MPi)	19.20	24.19
O_2 uptake (μgram atom O_2)	19.83	21.16
P:O ratio	0.99	1.14

Consequently ADP was used as phosphate acceptor to replace AMP which had been used for the initial studies.

Once the high energy compound ATP is formed, its breakdown to the acceptor molecules and inorganic phosphate by hydrolysis in the presence of an enzyme ATPase may readily

take place. Fluoride has been used to prevent the hydrolysis of ATP (24, 28, 50). However, when a glucose-hexokinase trapping system is used the need for fluoride is minimized (20). The effectiveness of the glucose-hexokinase trapping system was studied in considerable detail.

The trapping system was composed of glucose and the enzyme hexokinase in concentration that would not inhibit the oxidative activity. This was established by studying the effect of various concentrations of glucose and hexokinase on the rate of oxidation. However, 70 KM units of hexokinase per vessel were used because it gave optimal phosphorylation in initial studies. Glucose was used in concentration of 8μM per ml. reaction mixture. This gave the highest rate of oxidation as indicated by the following data.

Glucose Concentration	Rate - $Q_{O2}(N)$
0.010	323
0.008	356
0.0065	338
0.005	320
0.0025	317

In the presence of hexokinase, the energy trapped in the high energy bond of ATP is utilized to form glucose -6- phosphate (G6P) by the following reactions:

$$\text{ATP + glucose} \xrightarrow{\text{hexokinase}} \text{G6P and ADP}$$

The reactions involved in the phosphorylation scheme might be represented as follows:

$$\text{ADP} + \text{Pi} \xrightarrow[\text{phosphorylation}]{\text{enzyme succinate}} \text{ATP} \qquad 1.$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{G6P} \qquad 2.$$

It may be seen that the labelled phosphorus in the organic form should be in either ATP or G6P. If the trapping system was active, the inorganic phosphorus should be entirely in the G6P. Figure 6 clearly shows that G6P was formed according to equation 2. One of the difficulties encountered using the method described in Figure 6 was that high concentrations of glucose and G6P tended to run together. Therefore further studies of oxidative phosphorylation were carried out using Mortimer's (45) solvent. The separation of glucose and G6P by this method is shown in Figure 7.

Figure 8 presents the results of an experiment to determine if phosphorylation occurred according to equations 1 and 2. The reaction which contained ADP, Pi, enzyme, succinate, and the trapping system showed the formation of G6P. The control from which substrate (succinate) was omitted produced no detectable G6P on the chromatogram.

The effect of magnesium on oxidative phosphorylation is presented in table V. Some workers (10, 50, 59) reported that magnesium was required for oxidative phosphorylation The concentrations of magnesium used varied from 1µM to 10µM. In the studies carried out $MgSO_4$ was used at 1 µM per ml. reaction

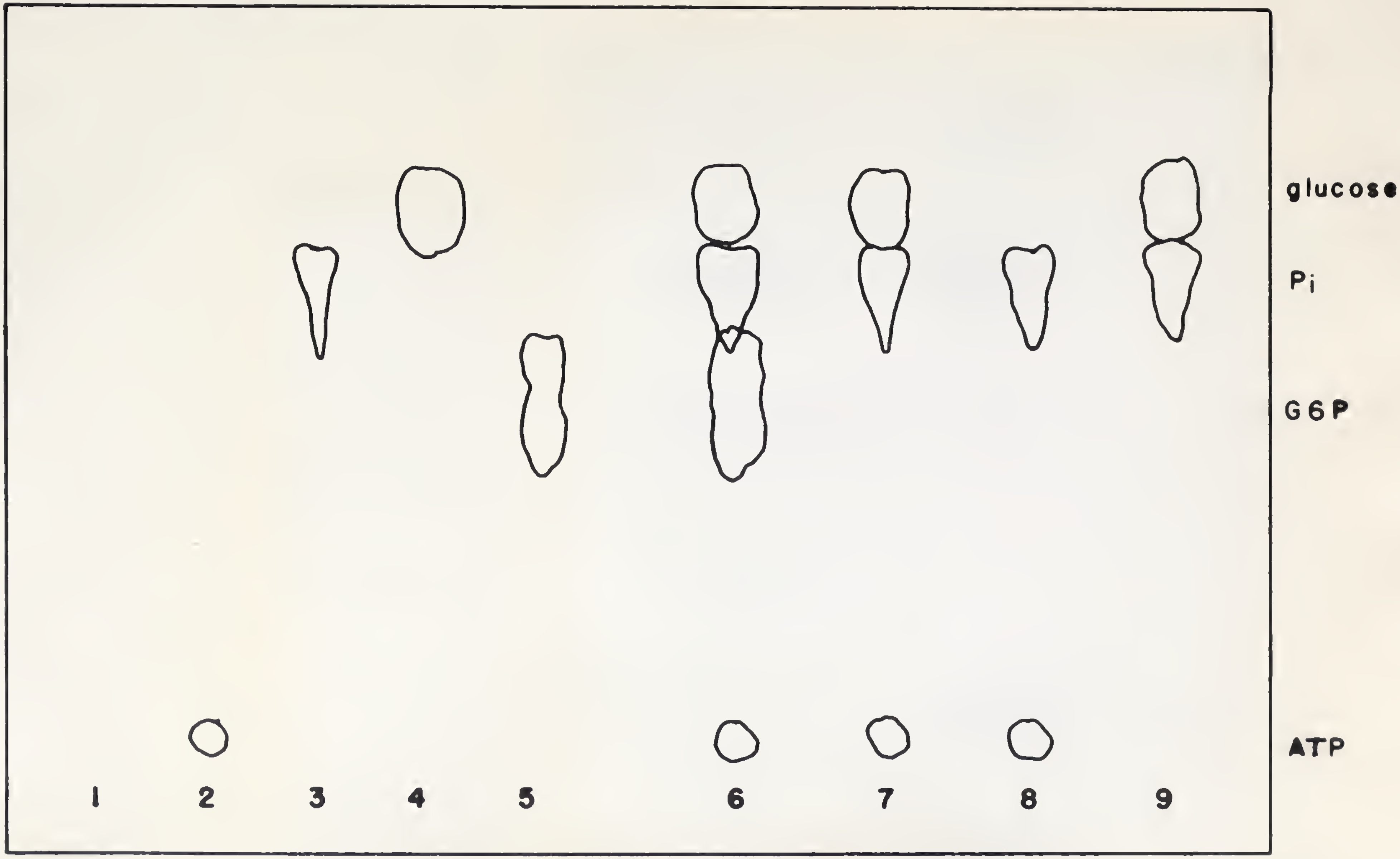


Fig. 6. Diagram of a chromatogram showing the separation of known compounds and mixtures of compounds to establish the activity of the trapping system. The solvent was methyl alcohol: formic acid: water (80:15:5) on Whatman #1 filter paper. Sprayed with both indicators. 1-hexokinase; 2-ATP; 3-Pi; 4-glucose; 5-G6P; 6-mixture of 1 to 4 (complete); 7-complete less hexokinase; 8-complete less glucose; 9-complete less ATP.

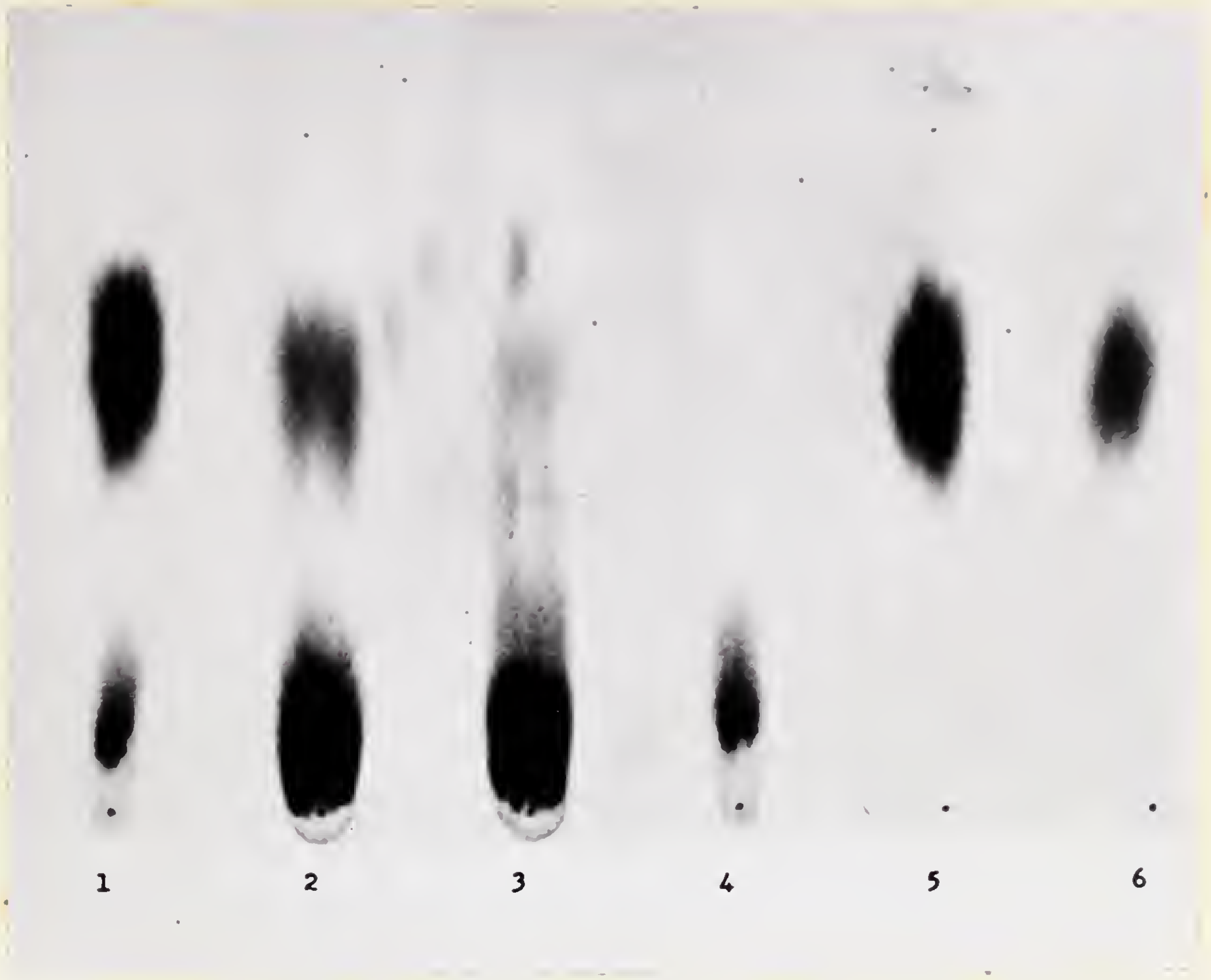


Fig. 7. Chromatogram of the separation of glucose and G6P with ethylacetate: acetic acid: water (3:3:1) solvent on Whatman #4 paper. Partridge's spray indicator was used. 1-10λ glucose, 2λ G6P; 2-2λ glucose, 10λ G6P; 3-10λ G6P; 4-2λ G6P; 5-10λ glucose; 6-2λglucose.

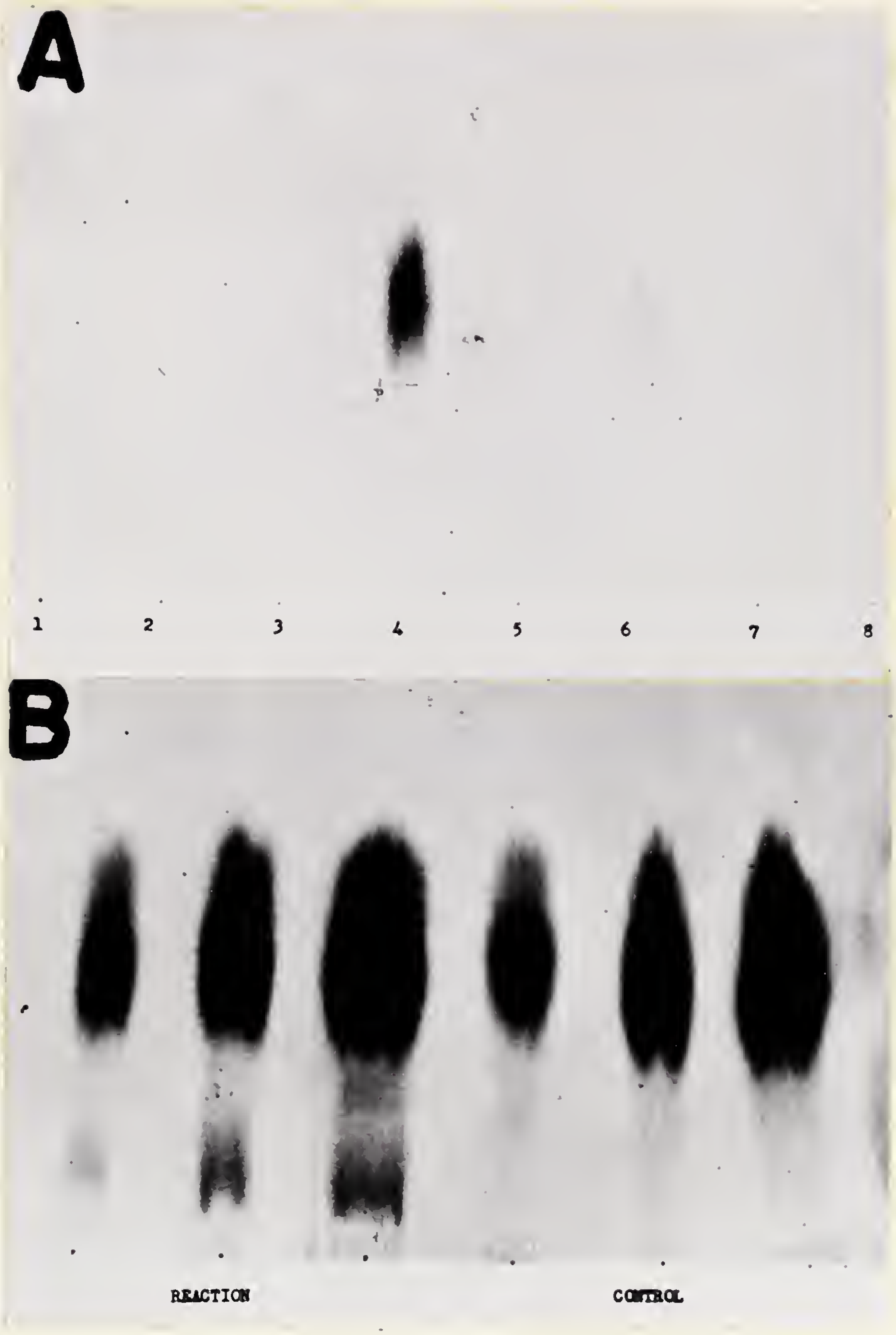


Fig. 8. Chromatograms showing active phosphorylation by the formation of G6P.

A. Chromatogram of the compounds present in the reaction mixture. 1 - $MgSO_4$; 2 - BSA; 3 - hexokinase; 4 - glucose; 5 - ADP; 6 - sucrose; 7 - phosphate buffer; 8 - succinate.

B. Chromatogram of reaction (substrate present) and control (no substrate) indicating the formation of G6P in the presence of succinate.

mixture. When magnesium was omitted, oxidation and phosphorylation were both inhibited.

Table V. Effect of magnesium upon oxidative phosphorylation.

	added Mg^{++}	minus Mg^{++}
Phosphorus esterified (μM Pi)	8.79	3.91
Oxygen uptake	13.9	9.4
P:O ratio	0.63	0.42

Components per ml. reaction mixture: 1μM ADP; 1μM $MgSO_4$; 20μM succinate: 22μM Pi; 88μM sucrose; 70 KM units hexokinase; 8μM glucose; 0.6 mg. BSA. Total volume 3.0 ml.

Table VIshows the results that were obtained when different concentrations of phosphorus were used in the reaction mixture. Increase in phosphorus concentration did not affect oxidation while phosphorylation was increased. A concentration of 66μM phosphorus per vessel was used since phosphorylation activity reached a maximum and oxidative activity was unaffected.

Table VI. Effect of phosphorus on oxidative phosphorylation.

Phorphurus Concentration (μM)	Phosphorus Esterified (μM Pi)	Oxygen Uptake	P:O Ratio
36	14.0	21.0	0.67
66	17.6	20.3	0.87
96	17.7	19.3	0.92

Reaction mixture as given in Table V. Phosphorus concentration as shown.

Pierpoint (50) and Switzer and Smith (59) also studied the effect of phosphorus concentration. They did not indicate the effect of phosphorylative activity. However, like the results reported here, Pierpoint obtained no effect on oxidation whereas Switzer and Smith reported an increase in oxidation with increased phosphorus concentration.

The results in table VII represent recovery studies on four reaction mixtures involving radioisotope. It may be seen that the number of counts recovered are very close to the counts added. The total number of counts added were reduced to keep loss due to coincidence counting as low as possible. When 80,000 cpm were added, approximately 10% of the counts were not accounted for. This loss was attributed to the very high count in the inorganic phosphorus precipitate and thus a high loss of counts by coincidence.

Table VII. Recovery of radioisotopic phosphorus in counts per minte.

	1	2	3	4
Total added	6310	4800	5320	4920
Amount recovered				
a) Organic portion	3260	2760	3200	2770
b) Pi precipitate	2180	1870	2170	2020
c) Wash from Pi precipitate	585	220	210	255
Total recovered	6025	4850	5580	5045
% loss or gain	-4	+1	+5	+3

Reaction mixture as in Table V .

Malonate

The effects of malonate upon the oxidative and phosphorylative activity of the succinoxidase system are shown in figure 9. The results were based on pea mitochondria from 1959 seed. At low malonate concentrations no effect was observed but an increase in concentration to 10^{-5} M resulted in an increase in phosphorylation and a very slight increase in oxidation. At a concentration of 10^{-2}M, oxidation and phosphorylation were almost completely inhibited. This may be taken as evidence for competitive inhibition (11).

Malonate has been used as a competitive inhibitor for succinic dehydrogenase on the assumption that it is inert metabolically. However, studies with mitochondria isolated from rat kidney (47), indicated that malonate may be oxidized through malonyl CoA to acetyl CoA and thus gain entry to the TCA cycle. Bentley (8) has reported the presence of malonate in leaves of numerous plant species including green wheat plants. Using peanut mitochondria, Giovanelli and Stumpf (25) reported that malonate in low concentrations was oxidized by the following pathway.

$$\text{Malonate} \xrightarrow{\text{CoA, ATP}} \text{Malonyl CoA} \longrightarrow CO_2 + \text{acetyl CoA} \rightarrow \text{TCA cycle}$$

The very slight increase in oxidation and larger increase in phosphorylation observed may indicate that here too malonate was oxidized to malonyl CoA and acetyl CoA. It seems likely that if acetyl CoA was formed, further cyclic oxidation would increase phosphorylation to a much greater extent than oxidation.

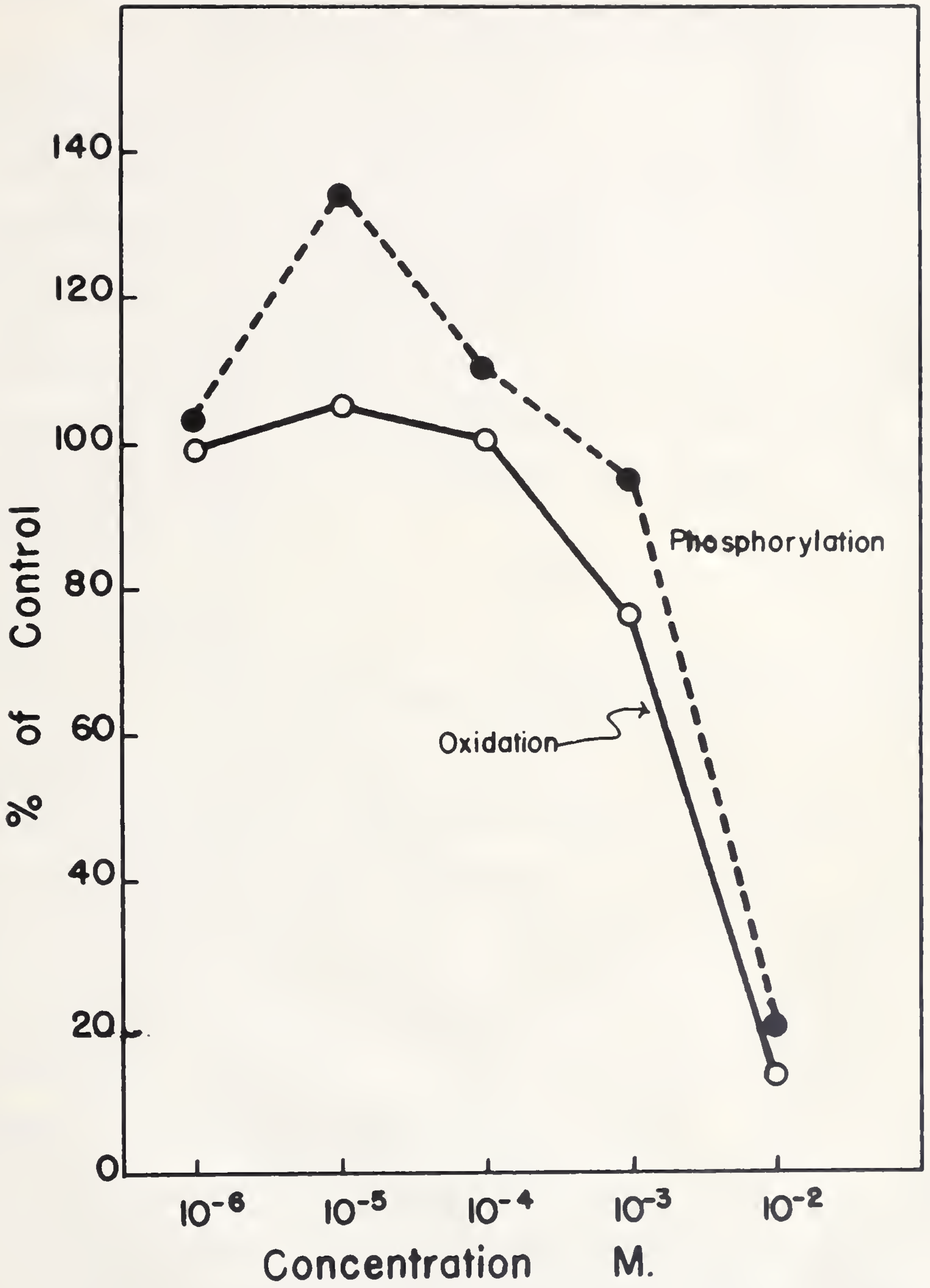


Fig. 9. Effect of malonate on oxidation and phosphorylation by pea mitochondria. The reaction mixture was as described in table V except 50 µM succinate was used.

2,4-Dinitrophenol

Dinitrophenol has been used extensively to uncouple phosphorylation from oxidation (40, 13, 33, 34). The effects of DNP on 1958 seed + BSA (20μM substrate) and 1959 seed + BSA (50μM substrate) were in close agreeement. These were averaged for presentation in figure 10. The DNP at 10^{-4} M inhibited phosphorylation without effecting the rate of oxidation. This clearly indicates the uncoupling of phosphorylation by DNP. The results obtained are in agreement with those of other workers (20, 28, 33, 53).

Maleic Hydrazide

Figure 11 shows that MH had paractically no effect upon oxidation and phosphorylation. If the action of MH was on the dehydrogenase system as reported by Isenberg et al.(31), there should have been inhibition of oxidation. Likewise, if its effect were based upon reaction with thiol groups of enzymes as proposed by Muir and Hansch (46), there should have been inhibition. The lack of inhibition suggests that MH does not affect the succinic dehydrogenase system. The results support the report of Weller et al.(64) that MH does not combine with sulfhydryl groups to cause enzyme inhibition. They also support the work of Baker (5) that MH does not affect the succinoxidase system.

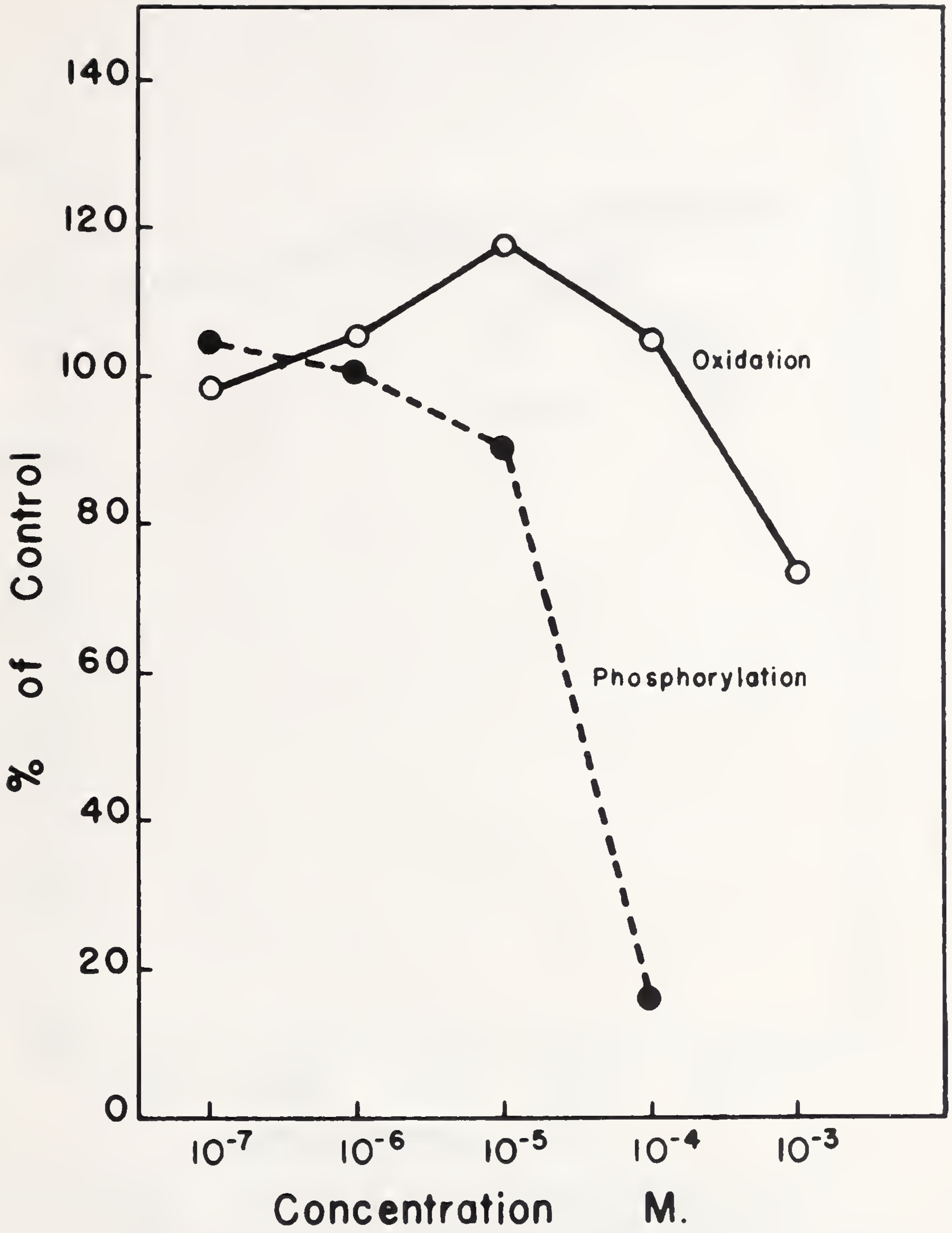


Fig. 10. Uncoupling of phosphorylation from oxidation by 2,4-DNP. Components of the reaction mixture were as described in table V.

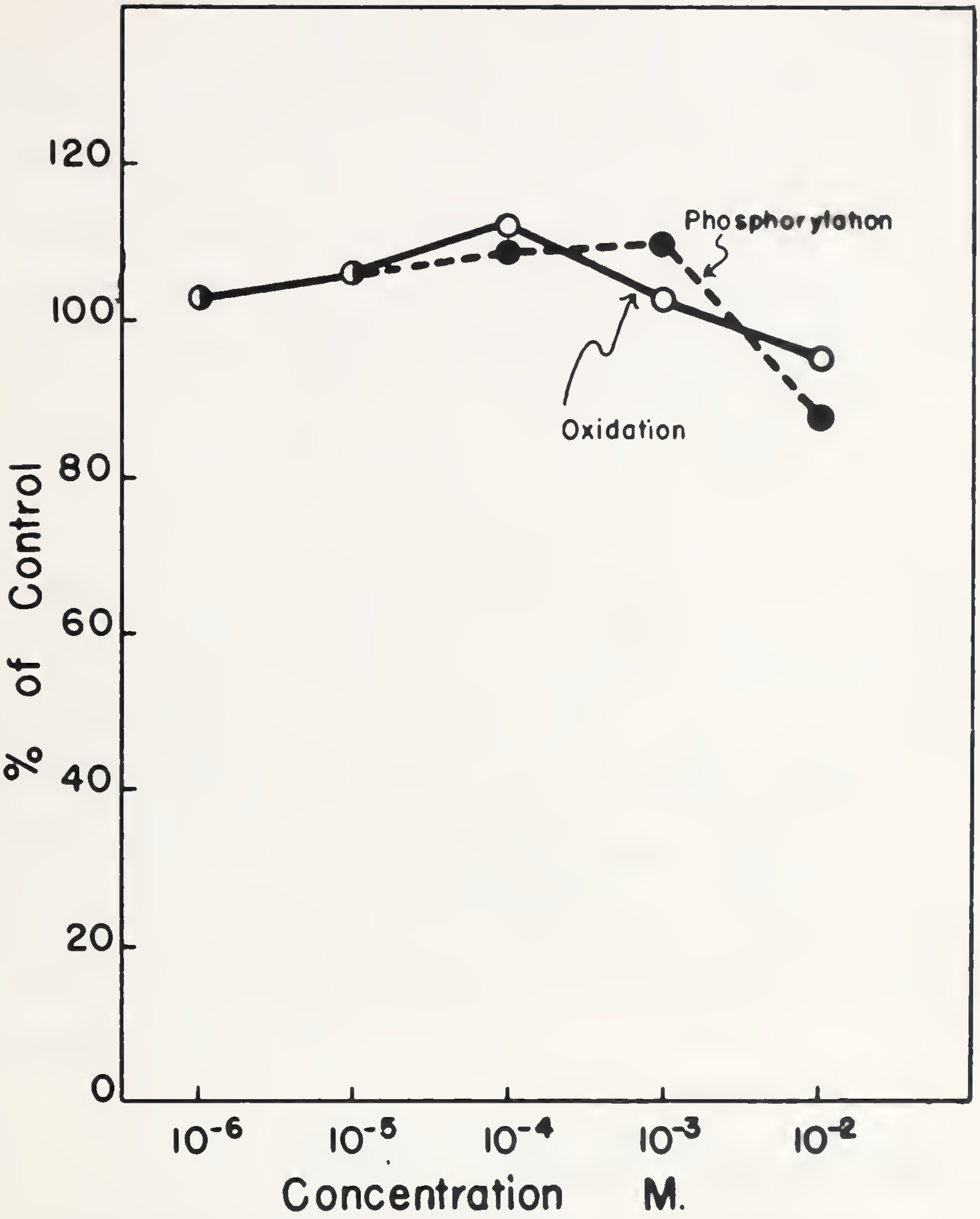


Fig. 11. The effect of maleic hydrazide on oxidation and phosphorylation by pea mitochondria. The results are for 1959 seed. Reaction mixture as in table V except that 50 μM substrate was used.

Indoleacetic Acid

The effects of IAA on 1958 seed + BSA (20 μM substrate) and 1959 seed + BSA (50 μM substrate) were averaged. It may be seen that IAA had no effect upon oxidation at any of the concentrations used (Fig. 12). There was slight inhibition of phosphorylation at the higher concentrations. Since phosphorylation is more labile than oxidation, addition of IAA might show a toxic effect on phosphorylation more readily than on oxidation.

Poljakoff-Mayber (52) working with mung-bean mitochondria, reported that IAA at 5 mg./L (equivalent to 2.9×10^{-5}M) had no effect on oxidative phosphorylation of a number of substrates including succinate. At 70 mg./L (4.1×10^{-4}M) oxidation was decreased due to toxic effects of IAA. Working with hypocotyls, Poljakoff-Mayber concluded that the IAA effect on growth was totally independent of TCA cycle enzymes. The results obtained with succinate, one of the TCA cycle acids, support this view.

NUMBER OF DETERMINATIONS PER EXPERIMENT

Table	Number of determinations	Figure	Number of determinations	Figure	Number of determinations
I	*	1	2	7	4
II	2	2	2	8	4
III	3	3	3	9	3
IV	2	4	2	10	2
V	3	5	3	11	3
VI	2	6	2	12	4
VII	2				

* all phosphorylation determinations were carried out as in table I.

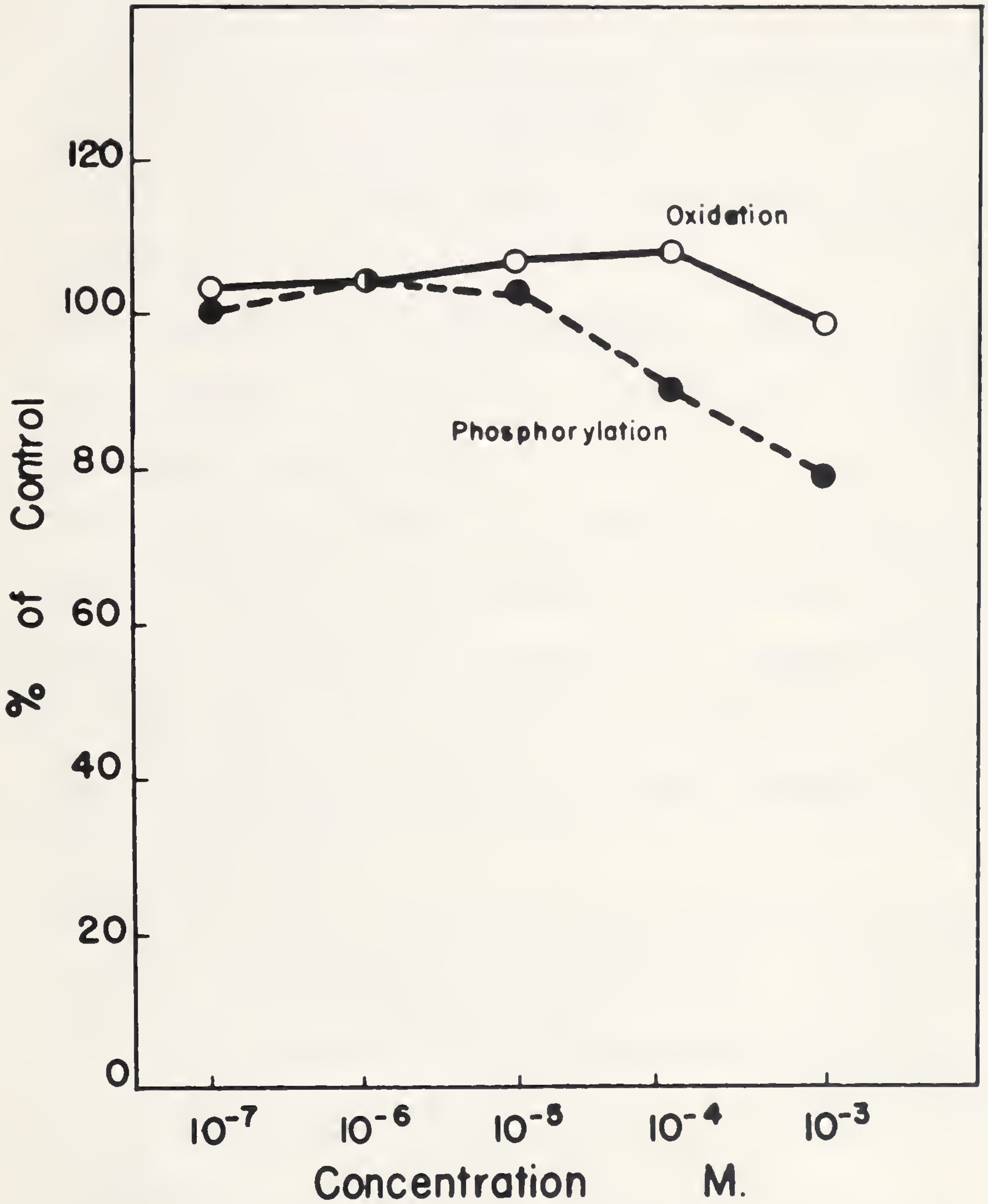


Fig. 12. The effect of indoleacetic acid on oxidation and phosphorylation using succinate as substrate. Reaction mixture as described in table V.

SUMMARY

1. A mitochondrial preparation obtained from pea seedlings exhibited oxidative and phosphorylative activity when succinate was used as substrate.
2. The activity of the succinoxidase system was maximal for three to four day old seedlings.
3. Bovine serum albumin used in the enzyme preparation increased oxidation.
4. High oxidative and phosphorylative activity was obtained by using optimum concentration of substrate, enzyme, magnesium phosphorus and glucose.
5. The high energy bond of ATP formed by phosphorylation was trapped in G6P in the presence of glucose and hexokinase.
6. The use of radioactive phosphorus combined with a procedure for the quantitative separation of inorganic and organic phosphates yielded a sensitive and simple method for measuring phosphorylation.
7. Malonate at high concentrations acted as a competitive inhibitor for succinic dehydrogenase. At low concentrations the slight increase in oxidation and larger increase in phosphorylation was assumed to be evidence for the oxidation of malonate.
8. 2,4-Dinitrophenol at 10^{-4} M uncoupled phosphorylation from oxidation.
9. Maleic hydrazide had practically no effect upon oxidation or phosphorylation when used at concentrations of 10^{-6} M to

10^{-2} M.

10. Indoleacetic acid at concentrations of 10^{-7} M to 10^{-3} M had no effect upon oxidation and phosphorylation.

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